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Reoviruses in Water Pollution Testing

Utah State Univ., Logan

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

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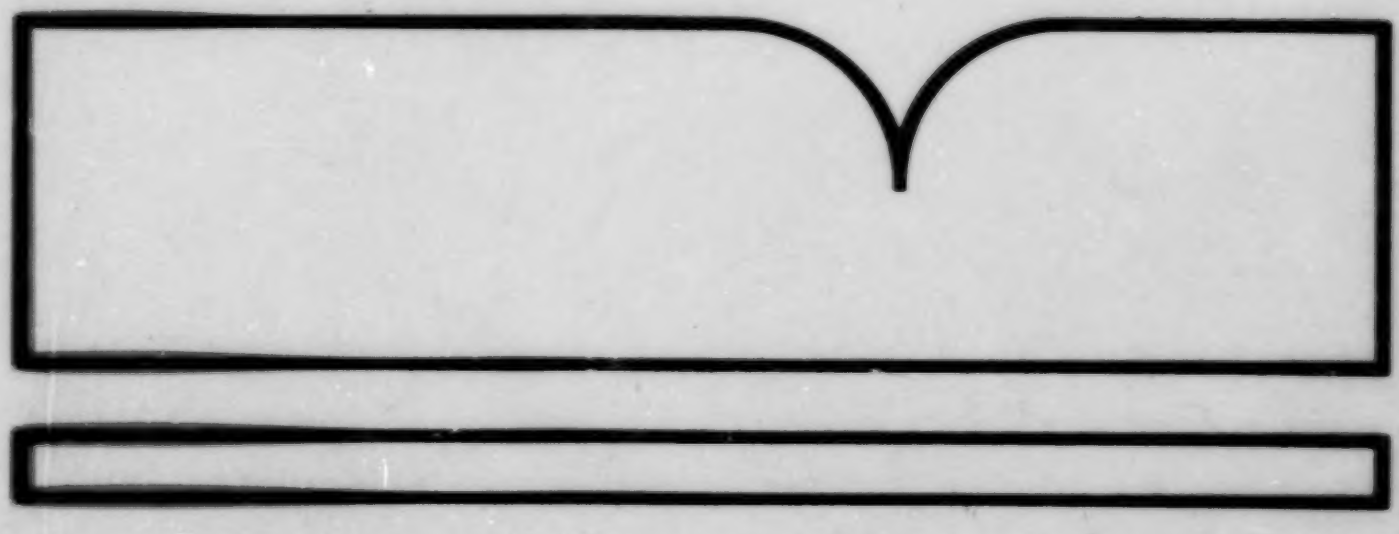

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16. ABSTRACT

Twelve cell lines were tested to determine their sensitivity to reoviruses of three serotypes that had been isolated from sewage. Madin-Darby bovine kidney (MDBK) cells were the most susceptible. Sewage-isolated, protamine-precipitated reoviruses were also used in conjunction with MDBK cells in a comparative evaluation of immunofluorescent cell count (ICC) and plaque assay procedures. Immunofluorescence assay is more sensitive and more rapid than plaque assay procedure for quantifying reoviruses recovered from sewage. A procedure for protamine sulfate precipitation of reoviruses and rotaviruses was developed. The use of egg albumin with protamine sulfate efficiently precipitates virus present in sewage and allows assay of reovirus by immunofluorescence. The virus-containing precipitate can be collected by low speed centrifugation rather than filtration, which allows larger sample volumes to be examined. The optimal concentration of enzyme for enhancing recovery of reoviruses and rotaviruses precipitated from sewage was 200 µg of trypsin/ml. During a one year period, the concentration of reoviruses was compared with the concentration of enteroviruses in sewage. The reoviruses were consistently recovered in approximately 5-fold higher concentrations than the enteroviruses. Reovirus infectivity is activated by enzyme treatment in some sewage, but not in other sewage. Some reoviruses recovered from sewage were inactivated by enzyme treatment.

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SECTION 1

INTRODUCTION

Enteric viruses are known to occur in water that receive sewage effluents and other wastewaters. There is, however, no assurance that the procedures currently being used for detecting viral pollution of water are adequate or are the most sensitive procedures available for quality control testing. If the public is to be protected from the hazards of viral disease posed by waterborne exposure, it is imperative that the best possible standard virus testing procedures be developed.

This report describes a study to evaluate reoviruses for their potential usefulness in water quality testing. The reasons for studying reoviruses were as follows: (a) Their abundance and consistent occurrence in sewage. (b) They are rarely known to cause disease, so are relatively safe to handle in the laboratory, and are unlikely to become targets of eradication by vaccination. Therefore, standardized tests based on reovirus detection will not have to be changed in the future. (c) They have a broad host range, so contamination by man and lower animals is detected. (d) They are amenable to low-cost isolation and identification procedures (e) A single antiserum will detect reoviruses of all three serotypes. (f) Reoviruses are easily cultivated to high infectious titers. (g) Reovirus hemagglutination inhibition tests can be used to assay antibodies in epidemiologic studies. (h) The vast majority of reoviruses are present in a potentially infectious form which is not normally detected by conventional infectivity assays (11, 22, 23). These assays detect only the infectious form unless the virus is exposed to proteolytic enzymes which remove the outer coat of the virus (22), and convert potentially infectious virus (PIV) to infectious virus (IV). The intact double coat of potentially infectious reoviruses makes them exceptionally resistant to inactivation (24). Consequently, if they are inactivated, other viruses present in water are likely to be inactivated. (i) The potential importance of enzyme enhanceable reoviruses as an indicator of viral pollution in water had never been assessed.

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REOVIRUSES IN WATER POLLUTION TESTING

by

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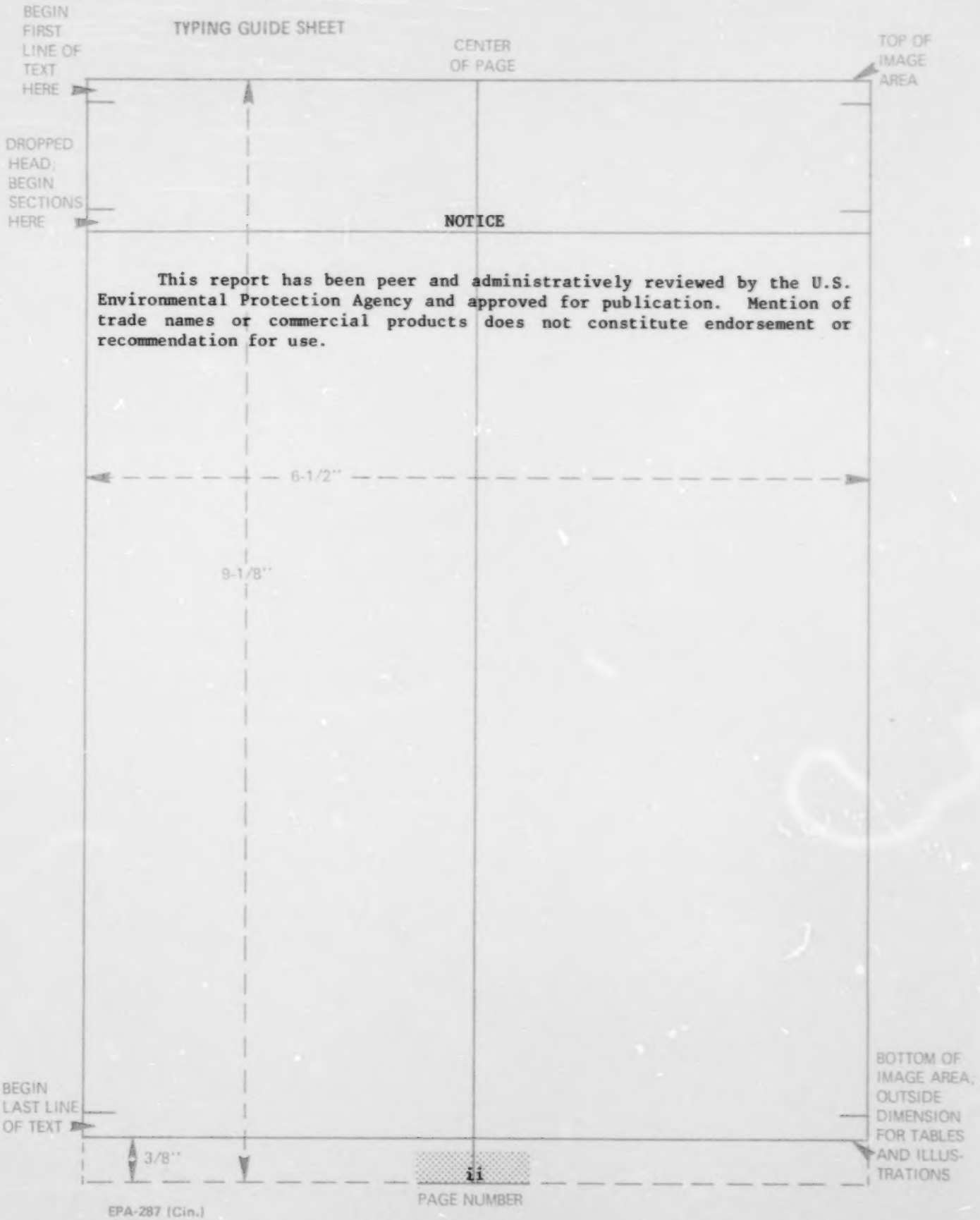
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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides, and nonionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

This report provides data on the occurrence of human reoviruses in sewage and surface water contaminated by sewage. Such studies are conducted in an effort to identify appropriate indicators of the occurrence of pathogenic viruses in environmental waters so as to assess the infectious disease hazard.

F. Gordon Hueter, Ph.D
Director
Health Effects Research Laboratory

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ABSTRACT

Removal of the outer reovirus coat using proteolytic enzymes greatly increases the infectivity of most reoviruses prepared in cell culture. The purpose of this study was to determine the potential importance of enzyme-enhanceable reoviruses in water quality control testing. Procedures were developed that would recover and quantify infectious and enzyme-enhanceable reoviruses in sewage.

Twelve cell lines were tested to determine their sensitivity to reoviruses of the three serotypes that had been isolated from sewage. Madin-Darby bovine kidney (MDBK) cells were the most susceptible. Sewage-isolated, protamine-precipitated reoviruses were also used in conjunction with MDBK cells in a comparative evaluation of immunofluorescent cell count (ICC) and plaque assay procedures. Immunofluorescence assay is more sensitive and more rapid than plaque assay procedure for quantifying reoviruses recovered from sewage.

A procedure for protamine sulfate precipitation of reoviruses and rotaviruses was developed. The use of egg albumin with protamine sulfate efficiently precipitates virus present in sewage and allows assay of reovirus by immunofluorescence. The virus-containing precipitate can be collected by low speed centrifugation rather than filtration, which allows larger sample volumes to be examined. The optimal concentration of enzyme for enhancing recovery of reoviruses and rotaviruses precipitated from sewage was 200 µg of trypsin/ml.

During a one year period, the concentration of reoviruses was compared with the concentration of enteroviruses in sewage. The reoviruses were consistently recovered in approximately 5-fold higher concentrations than the enteroviruses. Reovirus infectivity is activated by enzyme treatment in some sewage, but not in other sewage. Some reoviruses recovered from sewage were inactivated by enzyme treatment. Additional work is needed to fully explore the conditions for enzyme enhancement of reoviruses recovered from sewage. However, even without enhancement, reoviruses were isolated from raw sewage in sufficiently high numbers to suggest that reovirus recovery procedures should be included in the virological evaluation of sewage-polluted waters.

This report was submitted in fulfillment of R806527 by Utah State University under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from 4/1/79 to 6/30/81, and work was completed as of 6/30/81.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	-- American Type Culture Collection
BGM	-- Buffalo green monkey kidney
BHK-21	-- baby hamster kidney
BOD	-- biological oxidation demand
BSA	-- bovine serum albumin
BS-C-1	-- African green monkey kidney
BSS	-- balanced salt solution
CT	-- chymotrypsin
EPA	-- Environmental Protection Agency (U.S.)
FBS	-- fetal bovine serum
HEL	-- human embryonic lung
ICC	-- immunofluorescent cell count
intestinal 407	-- human embryonic intestinal
IV	-- infectious virus
KB	-- human carcinoma cells of the nasopharynx
L 929	-- derived from mouse normal subcutaneous aveolar and adipose tissue
MA-104E	-- rhesus monkey kidney
MDBK	-- Madin-Darby bovine kidney
MDCK	-- Madin-Darby canine kidney
MEM	-- minimal essential medium
PFU	-- plaque forming units
PIV	-- potentially infectious virus
PK	-- pig kidney
RD	-- rhabdomyosarcoma
Vero	-- African green embryonic monkey kidney

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SECTION 1

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SECTION 2

CONCLUSIONS

Madin-Darby bovine kidney (MDBK) cells, rhesus monkey kidney cells (LLC-MK_2) and human embryonic intestinal cells (Intestinal-407) were the most susceptible cell lines, respectively, for assaying reovirus serotypes 1, 2 and 3 previously isolated from sewage. The MDBK cells were the most sensitive when compared to LLC-MK_2 cells and Buffalo green monkey kidney (BGM) cells for assaying reoviruses that had been precipitated from sewage by protamine and had never been exposed previously to cells in culture.

The ICC method was found to be more sensitive, more specific and more rapid than the plaque assay procedure for assaying reoviruses isolated from sewage. The optimal inoculum size and centrifugation conditions were an inoculum of 0.25 ml centrifuged at 3,000 rpm for 30 minutes in a Sorvall microtiter plate rotor.

When some protamine sewage precipitates were assayed for reoviruses, the PIV were present in higher concentrations than the IV. It appears that we encountered some chymotrypsin negative (CT^-) mutants in sewage in our study. The CT^- reovirus mutants are inactivated by chymotrypsin treatment (23, 24). Some sewage appeared to convert PIV to IV. This would explain why it was not possible to enhance the infectivity of some reoviruses that had been extracted from sewage, i.e., the enhancement occurred while the virus was in the sewage.

During a one year period, the concentration of reoviruses was compared with the concentration of enteroviruses in sewage. The reoviruses were consistently recovered in approximately 5 fold higher concentrations than the enteroviruses.

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SECTION 3

RECOMMENDATIONS

1. Reoviruses should be included when a standard test procedure for detecting viruses in water is adopted since this study and previous investigations have found that reoviruses are consistently present in sewage.

2. For recovery of reoviruses from water, additional studies need to be conducted to determine the best procedure. The procedure should be inexpensive and trouble free and capable of processing large volumes of water.

3. The immunofluorescent cell count and MDBK cells should be used in the assay of sewage for reoviruses.

4. The recommendation regarding enzyme treatment of reoviruses in water quality testing is that additional studies be conducted. The effect of adding EDTA directly to the sewage at the time of collection to protect any CT^- mutants from inactivation by enzymes in the sewage should be determined. This procedure, unfortunately, would inactivate rotaviruses.

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SECTION 4

MATERIALS AND METHODS

OPTIMIZATION OF PROCEDURES FOR ISOLATION AND QUANTIFICATION OF REOVIRUSES IN SEWAGE

Cell Lines

All but one cell line used for this project was grown and maintained in 50 percent minimal essential medium (MEM)and 50 percent Leibovitz's-15 medium (Gibco) with 10 percent fetal bovine serum (FBS). The human embryonic lung (HEL) cells were grown and maintained in Dulbecco's medium with 10 percent lactalbumin hydrolysate and 10 percent heat inactivated FBS. The cell lines used, their passage number, and acquisition were as follows: Buffalo green monkey kidney (BGM) cells, passage 114 from the Environmental Protection Agency (EPA); baby hamster kidney (BHK-21) cells from the University of Utah; African green monkey (BS-C-1) cells, passage 66 from Flow Laboratories; human embryonic lung (HEL) cells, passage 18 from the American Type Culture Collection (ATCC); human embryonic intestinal (intestinal 407) cells, passage 276 from ATCC; human carcinoma cells of the nasopharynx (KB) from Flow Laboratories; Rhesus monkey kidney ($LLC\text{-}MK_2$) cells, passage 10 from ATCC; Rhesus monkey kidney (MA-104E) cells, passage 52 from Dr. Estes at Baylor University; Madin-Darby bovine kidney (MDBK) cells, passage 105 from the United States Department of Agriculture, National Animal Disease Laboratory, Ames, Iowa; Madin-Darby canine kidney (MDCK) cells, passage 63 from Flow Laboratories; rhabdomyosarcoma (RD) cells from the EPA; and African green embryonic monkey kidney (Vero) cells, passage 158 from Microbiological Associates.

Viruses

Reoviruses used in the investigation to determine the sensitivity of various cells to reoviruses were isolated from sewage, serotyped by hemagglutination inhibition and assayed for infectivity in 12 different cell lines using an immunofluorescent cell counting procedure (20). Type 1 (strain OC7-88) and type 2 (strain OC7-51) reovirus sewage isolates were obtained from Nathalie Schmidt of the California State Department of Public Health in Berkeley. Both of these viruses were isolated in MK_2

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cells. Type 3 (strain TC-505), obtained from Dr. Beatrice England of the Department of Public Health, San Diego, California, had been cultivated in BGM and BS-C-1 cells. Previous to the experiment, the viruses had been assayed, diluted, aliquoted in MEM without FBS in 0.2 ml quantities and frozen at -20°C.

In other experiments, the protamine sulfate precipitation method was used to obtain reoviruses from raw sewage collected at the sewage treatment plant in Hyrum, Utah. These viruses had never been grown in cell cultures prior to use in this investigation.

Plaque Assay Procedure

The plaque assay, performed on MDBK, LLC-MK_2, and BGM cells was a modification of the procedure used by Dahling et al, (3). The cells were grown to 90-100 percent confluency in 25 cm^2 flasks (Falcon). After washing with Earle's balanced salt solution (BSS), 0.2 ml of the viral dilution was added to the cell sheets and adsorbed for one hour at 37°C. Following incubation, the infected cultures were overlaid with 5 ml of medium. Each 100 ml of the overlay medium contained: 41.5 ml of 2X MEM, 2.0 ml of FBS, 2.0 ml of 7.5 percent $NaHCO_3$, 1.0 ml of 1.0 percent $MgCl_2.5H_2O$, 1.0 ml of sterile (autoclaved) skim milk, 50 units/ml of penicillin-G (Sigma), 100 µg/ml streptomycin sulfate (Sigma), 50 µg/ml gentamicin (Schering), 1.0 µg/ml fungizone (GIBCO), 2X nonessential amino acids (GIBCO), and 50 ml of 1.2 percent Ionagar No. 1 (Oxoid) solution. Pancreatin (Oxoid) was added to the overlay medium to enhance reovirus plaque formation (25). A solution prepared by dissolving one pancreatin tablet in 50 ml of distilled H_2O at 37°C was sterilized using a 0.2 µm pore size membrane filter. The stock solution was stored at -20°C and diluted 1:70 in the overlay medium. After 3 days, 2 ml of the above medium containing 0.003 percent neutral red was added as a vital stain; the plaques were counted 3-5 days after the cells were infected.

Immunofluorescent Cell Count (ICC) Assay

The ICC assay performed throughout this project is the method of Spendlove (20). The stock cells were trypsinized, centrifuged, and resuspended in MEM without fetal bovine serum to approximately 1 x 10^6 cells/ml. The cell suspension was seeded onto 15 mm circular glass coverslips (3 per 60 mm petri dish) in aliquots of 0.2 ml, allowed to attach, and incubated at 37°C for one hour. Subsequently, 5 ml of growth medium with fetal bovine serum was added per petri dish. When the cell sheets had a uniform density over the surface and were confluent near the edge, the coverslip cultures were washed with Earle's BSS and infected.

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Twenty microliters of virus inoculum was added to each cell sheet, the cultures were incubated at 37°C for approximately one hour, after which time growth medium without FBS was added. At the end of 20-22 hr of incubation, the maintenance medium was removed and 5 ml of cold acetone (4°C) was added for 5 min. The infected cells were then stained for one hour with antireovirus fluorescent antibody (20), and washed for 10 min in distilled-deionized water. When the cultures had dried, they were mounted in saline:glycerol (1:10) and the infected cells were counted using a fluorescence microscope.

In the ICC procedure, it is not necessary to count all of the cells on a coverslip. If only part of the coverslip is scanned, an appropriate multiplication factor is used. The following list shows the multiplication factors used with the different objective lenses and information used in calculating the factors.

	Objective Lenses		
	10X	25X	40X
Diameter of field (mm)	1.78	0.71	0.45
Useful diameter for scanning (mm)	1.75	0.70	0.44
Area of a single field (mm^2)	2.49	0.40	0.12
Multiplication factor for a single field	70.6	445	1100
Area of a single microscopic scan through the center of a 15 mm coverslip culture (mm^2)	26.2	10.5	6.6
Multiplication factor for a single scan	6.7	16.8	26.2

Area of 15 mm coverslip = πr^2 = 176.7 square millimeters.

If 0.02 ml of a 10^{-2} dilution of virus were assayed and 90 fluorescing cells were counted in a single scan through the center of a culture using a 10X objective lens the infectivity count would be 3.0 X 10^6 viruses/ml. The factors used in making this calculation are as follows:

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X50 - a 0.02 ml inoculum was used.
0.02 = 1/50 of a milliliter.
X100 - the virus dilution assayed was 10^{-2}.
X90 - 90 fluorescing cells were counted.
X6.7 - Multiplication factor for a single scan using a 10X objective.

$50 \times 100 \times 90 \times 6.7 = 3.0 \times 10^6$ viruses/ml

All assays were performed in triplicate. The tests for nonspecific staining have been described previously (20).

Duncan's Multiple Range Comparison Test

The data from these studies were statistically analyzed using the "Duncan Multiple Range Test" (13). The data were obtained using a biological assay procedure so they were inherently variable. The Duncan Test was used, therefore, to objectively corroborate the differences in the results. The Duncan Test incorporates probability and degrees of freedom to determine if significant differences exist between the various treatments. The computer analyses were interpreted by noting which treatments share a column of asterisks. In the present tables, those treatments which do not share a common number are to be interpreted as "significantly different" at a 95 percent confidence level.

PROTAMINE PRECIPITATION OF TWO REOVIRUS PARTICLE TYPES

Virus and Cells

Plaque-purified type 1 (Lang strain) reovirus was used in the protamine precipitation experiments. Virus stocks were prepared in roller bottle or suspension cultures of mouse L 929 cells. Dense cell layers were incubated for about 1 hour at 37°C with approximately 20 viral particles per cell. The basal medium of Eagle (Gibco) containing antibiotics, and 2 percent FBS was adjusted to pH 6.8 to 7.0 using sodium bicarbonate. This medium was added to infected cells. After incubation for 24 hours at 37°C the infected cells were frozen and thawed six times, and centrifuged to remove cell debris. The supernatant fluids were pooled and stored at -20°C in 2 ml aliquots.

Enzyme Treatment and Virus Assay

Enhancement of the infectivity of reoviruses with chymotrypsin (Worthington Biochemical Co.) has been described (23,24). Viral preparations were exposed to 20 to 200 ug of chymotrypsin per ml at 37°C for 1 hour. Viral ICC assays were performed both before and after chymotrypsin treatment using MDBK cells. Results of

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the assays were determined by averaging counts taken from three coverslip cultures at each dilution.

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Concentration Procedures

England's (4) procedure for concentrating viruses from sewage and effluents is as follows. Prior to water or sewage filtration, tween 80 was passed through all prefilters (Millipore Corp., #AP20) to prevent subsequent viral adsorption to the filter discs (26). Tween 80 treated prefilters alone were used for filtration of the sewage samples and for collection of the protamine precipitate. Sample volumes of sewage ranging from 200 to 2,000 ml were filtered through a double AP20 disc by vacuum. Bovine serum albumin was then added to the filtrate to a final concentration of 0.25 percent. The albumin-supplemented sample was adjusted to pH 7.5 to 7.8 with 1N HCl or 1N NaOH. A 1 percent stock solution of protamine sulfate (National Biochemicals Corp.) was added to the sample to a final concentration of 0.05 percent for the sample's first precipitation, and 0.025 percent for the second. The solution containing protamine and the sample was stirred for 30 minutes at room temperature.

The precipitate was collected by passing the sample through a Millipore AP20 disc under negative pressure. After a second precipitation and filtration, the two precipitates on the upper surface of the AP20 discs were dissolved and collected in a test tube in the following manner. With the vacuum off, a small volume of 1M NaCl (usually 0.5 ml) was pipetted over the surface of the AP20 disc, allowed to soak for about 5 minutes to dissolve the precipitates, and then drawn into the receiving tube using vacuum. Sterile water, six times the volume of the 1M NaCl, was pipetted over the surface of the prefilter disc and drawn into the test tube to produce an isotonic filtrate. Fetal bovine serum, 10 percent by volume, was added to stabilize the virus. The concentrated filtrate was centrifuged at 2,500 rpm for 30 minutes to sediment bacteria. The supernatant fraction was inoculated onto cell cultures (4). The concentration factor for the inoculated virus precipitate ranged from 100-500 fold.

Modifications of the above procedure, to concentrate and assay the IV and PIV were as follows. (a) Fetal bovine serum low in reovirus antibody, as determined by a neutralization test, was used instead of bovine serum albumin, at the same (0.25 percent) concentration. (b) The protamine sulfate concentration was reduced to 0.005 percent for the first precipitation and 0.0025 percent for the second because we found higher protamine and FBS concentrations interfered with enzymatic enhancement of viral infectivity and with the fluorescent cell assay. (c) Two equal volumes of of 1M NaCl, with the total volume equaling 0.5 to 1.5 ml, were used to recover virus from the protamine precipitates. (d) After centrifuging to remove any bacteria present in the precipitated sample, the supernatant was assayed on coverslip cultures

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using ICC procedure before and after chymotrypsin treatment of the virus concentrate. All results represent the average of two to four experiments involving river water samples containing 500 to 4,000 ml.

River Water

Class A* water was collected from the Logan River, and filtered through a 0.22 μm pore size Millipore filter to remove suspended matter, and then inoculated with reovirus. If the water samples were not filtered, 10 to 30 percent of the virus adsorbed to the suspended solids could not be recovered after precipitation.

Viral Aggregation

It has been shown that reoviruses aggregate when diluted less than 100 fold into distilled water (5). This aggregation is concentration dependent, not happening at dilutions of 100 fold or greater. Therefore, 0.1 to 1.0 ml of stock virus was added to 500 to 4,000 ml of filtered river water containing 0.25 percent FBS at a pH between 7.5 and 7.8. If unusual differences were observed between the infectivity titers of stock virus and river water inoculated with reovirus (the control), the experimental results were discarded.

Sonication

Several suspensions of reovirus in river water were sonicated to disrupt viral aggregates. Reovirus that had not been enzyme treated was diluted into 500 ml of filtered river water that contained 0.25 percent fetal bovine serum at a pH between 7.5 to 7.8 and was mixed for 5 minutes. A 15 ml sample of the viral suspension was withdrawn, placed in a glass tube in an ice bath, and sonicated with the small probe of the Bronwill Biosonik III. Samples were sonicated repeatedly at a maximum sonicator setting for 15 seconds for a total of 3.5 minutes. After every 30 seconds of sonication, a 1 ml sample was removed and assayed for IV and PIV.

Sewage Samples

Raw sewage samples were collected from Salt Lake City and Dugway, Utah. Salt Lake City sewage, collected at the north treatment facility is greatly diluted with drainage and industrial waste water. Dugway sewage dilution is restricted to household water. Samples from both cities were collected at the point of inflow

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*Class A water is that designated for protection of cold water species of game fish, water fowl and agricultural use. In part, the quality restrictions require a BOD <5 ml/1, NO_3 <4 mg/1, and PO_4 <0.25 mg/1 (Dr. Dennis George, personal communication).

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before treatment. Grab samples with volumes between 1,000 and 2,000 ml were taken for testing.

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A PROTAMINE SULFATE CONCENTRATION METHOD FOR REOVIRUSES AND ROTAVIRUSES ADAPTABLE TO LARGE WATER VOLUMES

Virus and Cells

Plaque purified type 1 (Lang strain) reovirus and bovine rotavirus were used in all experiments. Reovirus was produced in mouse L-929 cell suspension or roller bottle cultures maintained in MEM (Joklik modified; GIBCO, Grand Island, N.Y.), supplemented with 2 to 5 percent FBS. After 24 hr infection, the suspension cultures were centrifuged to concentrate the infected cells.

Bovine rotavirus stocks were prepared in roller bottle cultures of MDBK cells. Cell monolayers were washed several times with MEM without FBS followed by virus adsorption for 1 hour. MEM containing antibiotics was adjusted to pH 6.8 to 7.0 using sodium bicarbonate and added to the infected cells. After 18 to 22 hours at 37°C, the infected cells were scraped from the bottle and centrifuged to pellet the infected cells and cell debris.

Both reovirus and rotavirus were partially purified using fluorocarbon (Freon 113; Rakon; Wichita, KS). Equal volumes of cell debris and Freon were homogenized in a mixer. The preparations were centrifuged and the viral-containing aqueous (upper) layers were collected. Numerous viral concentrations were pooled to form single lots of both viruses which assayed at approximately 1×10^{10} ICC/ml for reovirus and approximately 9×10^{8} ICC/ml for rotavirus. Each virus preparation was divided into aliquots and stored at -85°C until needed.

Enzyme Treatment and Virus Assay

Enhancement of the infecticity of reovirus and rotavirus preparations with proteolytic enzymes was performed as previously described (p. 7). Trypsin was neutralized by addition of Soybean trypsin inhibitor (Sigma Chemical Co; St. Louis, MO) at 1.5 times the trypsin concentration used before inoculation onto cell cultures. At trypsin concentrations below 20 µg/ml of sample, this neutralization was not necessary. Viral infectivity was assayed using the ICC assay.

Concentration Procedures

Studies were conducted to simplify the protamine precipitation technique to improve concentration of larger volumes and permit rapid assay of reovirus and rotavirus IV/PIV particles.

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Modificiations of England's protamine sulfate precipitation (see pg. 8) to concentrate and assay IV and PIV reovirus particles included: (a) Use of egg albumin as the floc facilitator. (b) Reduction of the protamine sulfate concentration 10 fold to 0.005 percent for the first precipitation and 0.0025 for the second. Higher protamine concentrations interfered with enzymatic enhancement of reovirus infectivity and with the fluorescent cell assay. (c) Step-wise elution of the virus-protamine precipitate from treated AP20 pre-filters with two equal volumes of 1 M NaCl. (d) Assay of reovirus concentrates on coverslip cultures by a fluorescent antibody procedure, before and after enzymatic enhancement of the reovirus concentrate. Protamine sulfate and Grade III egg albumin were obtained from Sigma Chemical Co. (St. Louis, MO) and prepared in separate stock solutions of 1 percent protamine sulfate and 2 percent egg albumin for use in experiments. Grade III albumin stock solutions consistently precipitated virus. Stock solutions of protamine and albumin were prepared twice a week and stored at 4°C until used.

Water and Sewage Samples

Chlorine free water was obtained from the Utah Water Research Laboratory.

Secondary sewage samples were collected at Hyrum, Utah sewage treatment plant just before the chlorination step. These samples were routinely low in suspended solids, i.e., they contained from 10 to 20 mg per liter. Secondary sewage samples found to lack naturally occurring reovirus and rotavirus were used as control samples. These samples were spiked with virus and precipitated at the same time as raw sewage as a check on the precipitation efficiency of the procedure.

Raw sewage samples were collected from the Hyrum treatment facility at the point of entry between 10:30 a.m. and 12:30 p.m. Sewage grab samples (0.5 l to 1.5 l) were immediately transported to the Utah State University, precipitated with the protamine sulfate procedure, and assayed for reovirus IV/PIV particles.

COMPARISON OF THE CONCENTRATIONS OF REOVIRUSES AND ENTEROVIRUSES IN SEWAGE

Cells

MDBK cells and BGM cells were used in this study. MDBK cells were grown in Eagle's MEM supplemented with 10 percent heat-inactivated

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FBS, 0.19 percent $NaHCO_3$, 50 units/ml penicillin-G (Sigma) and 100 µg/ml streptomycin sulfate (Sigma). BGM cells were grown in 50 percent MEM and 50 percent Leibovitz's-15 medium containing the same supplements as described above.

Virus

"Spiked" sewage samples were flocculated in parallel with the experimental sewage sample in order to measure the efficiency of the beef extract recovery method. Reovirus type 3, cultured in MDBK cells and poliovirus type 1, cultured in BGM cells were Freon extracted, aliquoted and frozen at -90°C. Both viruses were "spiked" at a concentration of approximately 1×10^6 virus particles/250 ml.

Beef Extraction (organic flocculation) of Virus from Sewage

The procedure of Katzenelson et al. (9), obtained from Dr. John L. Riggs of the California State Department of Public Health, was as follows: (a) A one liter sample of raw influent was centrifuged at 170 x g for 15 minutes to remove solids. Beef extract (Oxoid-Lab Lemco powder) was added to a 1 percent concentration. (b) The sample was divided into 3 volumes: 1) a 500 ml sample used for endogenous virus recovery, 2) a 250 ml "spiked" reovirus sample, 3) a 250 ml "spiked" poliovirus sample. (c) The pH of the solutions was reduced to 3.5 by the addition of 1.0 N HCl which results in the formation of an organic floc. (d) The flocs were allowed to settle (overnight at 4°C) and the majority of the clear supernatant was decanted and discarded. The flocs were then recovered by centrifugation at 2,000 x g for 15 minutes. (e) The pellets were dissolved in 25 ml of 0.15 M Na_2HPO_4 and the resultant preparation was diluted in MEM without FBS and assayed for viruses in cell cultures.

Immunofluorescent Microtiter Plate Assay

Confluent monolayers of MDBK cells in 24-2311 microtiter plates (Falcon) were washed twice with MEM without FBS and infected with 0.5 ml of diluted sewage extract. The infected cells were centrifuged at 2,000 rpm in a Sorvall GLC-1 tabletop centrifuge for 1/2 hour at room temperature and then placed in a 37° CO_2 incubator for 20-22 hours. Infections were stopped by aspirating the maintenance medium, allowing the cell sheets to dry and fixing with acetone diluted in PBS (1:5) for 1-2 minutes. Fixed cell sheets were allowed to dry and then stained overnight at room temperature or for 2 hours at 37°C with fluorescein-conjugated antireovirus antibody. After placing a drop of PBS in glycerol (1:10) in each well, a 15 mm circular glass coverslip was placed over the stained cell sheets. Infected (fluorescing) cells were counted on a Zeiss GFL eipfluorescence microscope. The procedures used to show that the fluorescent antibody staining was specific have been described previously (20).

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Plaque Assay Procedure

The plaque assay, performed on BGM cells, was a modification of the procedure used by Dahling et al.[2] (3). The cells were grown to 90-100 percent confluency in 25 cm^2 tissue-culture petri dishes (Falcon), and, after washing with Earle's BSS, 0.25 ml of the diluted sewage extract was added to the cell sheets and adsorbed for one hour at 37°C. Following incubation, the infected cultures were overlaid with 5 ml of medium. Each 100 ml of the overlay medium contained: 22 ml of 2X MEM, 22 ml of 2X Leibovitz's-15 medium, 3 ml of FBS; 2 ml of 7.5 percent $NaHCO_3$, 1 ml of 1 percent $MgCl_2.5H_2O$, 50 ml of 1.7 percent agar (BBL), and antibiotics as indicated on page 5. After 24 hours, the infected cell cultures were overlayed with 5 ml of the above medium containing 0.003 percent neutral red as a vital stain. Plaques were counted 24 to 48 hours after the second overlay.

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SECTION 5

RESULTS AND DISCUSSION

SENSITIVITY OF 12 CONTINUOUS CELL LINES TO SEWAGE-ISOLATED REOVIRUS.

The results (Tables 1-4) indicated that the MDBK cells were the most sensitive cells for assaying reovirus type 1 (Table 1). The infectivity assays using MDBK cells were 2 logs higher than assays in HEL cells, the least sensitive cells. Compared to MDBK cells, the sensitivity of LLC-MK_2 cells was 54 percent, intestinal 407 cells was 40 percent, and BGM cells was 35 percent. The MDCK, MA-104E, Vero, BS-C-1, PK, BHK-21, RD, and HEL cells were less than 20 percent as sensitive as the MDBK cells to reovirus type 1.

The LLC-MK_2, intestinal 407, and BGM cells were all significantly less sensitive than MDBK cells by Duncan's Multiple Range comparisons.

TABLE 1. SENSITIVITY OF 12 CONTINUOUS CELL LINES TO SEWAGE ISOLATED REOVIRUS TYPE 1

Cell line	Average Cell Count and Standard Deviation[a]	Infectivity titer/ml[b]	Duncan's Mult. Range Compar.[c]
MDBK	337 ± 27	2.8×10^6	1
LLC- MK_2	181 ± 18	1.5×10^6	2
INT-407	132 ± 37	1.1×10^6	3
BGM	111 ± 24	9.3×10^5	4
MDCK	66 ± 32	5.5×10^5	5
MA-104E	60 ± 5	5.0×10^5	5
VERO	44 ± 4	3.7×10^5	6
BS-C-1	43 ± 8	3.6×10^5	6
PK	39 ± 9	3.3×10^5	6
BHK-21	15 ± 6	1.2×10^5	7
RD	5 ± 3	4.2×10^4	7
HEL	4 ± 2	3.4×10^4	7

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[a]Average fluorescent cell count represents the average of a single scan through the center of six different coverslip cultures.
[b]Virus assayed by the ICC procedure.
[c]Same number ranking indicates no significant difference at a 95 percent confidence interval.
ICC Assay Multiplication Factors:
X10 - The viruses were diluted to fold to obtain quantitative counts
X16.8 - Factor for a single scan through the center of the coverslip culture.
X50 - 0.02 ml inocula were used (0.02 = 1/50).

Reovirus type 2 (Table 2) results were similar to those obtained with reovirus type 1. The same four cell lines were the most sensitive. LLC-MK_2, intestinal 407, and BGM cells were 80, 73, and 64 percent as sensitive as MDBK cells to reovirus type infection, respectively.

TABLE 2. SENSITIVITY OF 12 CONTINUOUS CELL LINES TO SEWAGE ISOLATED REOVIRUS TYPE 2.

Cell Line	Average Cell[a] Count and Standard Deviation	Infectivity titer/ml	Duncan's Mult.[c] Range Compar.
MDBK	249±37	2.1×10^8	1
LLC - MK_2	199±37	1.7×10^8	2
INT-407	182±17	1.5×10^8	3
BGM	160±12	1.3×10^8	4
MA-104E	124±14	1.0×10^8	5
VERO	100±16	8.4×10^7	6
BS-C-1	94±11	7.9×10^7	6
BHK-21	56±17	4.7×10^7	7
MDCK	51±12	4.3×10^7	7,8
PK	41±4	3.4×10^7	8
HEL	4±2	3.4×10^6	9
RD	1±2	8.4×10^5	9

[a]Average fluorescent cell count represents the average a single scan through the center of six different coverslip cultures.
[b]Virus assayed by the ICC procedure.
[c]Same number indicates no significant difference at a 95 percent confidence level.
ICC Assay Multiplication Factors:
$X10^3$ - The viruses were diluted 1000 fold to obtain quantitative counts.

X16.8 - Factor for a single scan through the center of the coverslip culture.
X50 - 0.02 ml of inocula were used (0.02 = 1/50).

The data obtained for type 3 reovirus (Table 3) differed little from those obtained with types 1 and 2 reovirus. The three most sensitive cell lines were LLC-MK_2, intestinal 407; however, the differences were not as distinct as with types 1 and 2. LLC-MK_2 and MDBK cells did not differ significantly in their sensitivity. Intestinal 407 had 86 percent as many infected cells as the LLC-MK_2 cells.

TABLE 3. SENSITIVITY OF 12 CONTINUOUS CELL LINES TO SEWAGE ISOLATED REOVIRUS TYPE 3.

Cell Line	Average Cell Count and Standard Deviation[a]	Infectivity titer/ml[b]	Duncan's Mult. Range Compar.[c]
LLC-MK_2	123±13	1.0×10^7	1
MDBK	118±10	9.9×10^6	1
INT-407	106±34	8.9×10^6	2
MDCK	98± 8	8.2×10^6	2,3
BHK-21	93±18	7.8×10^6	3
MA-104E	72±11	6.1×10^6	4
PK	27± 4	2.3×10^6	5
BGM	21± 5	1.8×10^6	5,6
VERO	16± 4	1.3×10^6	6
BS-C-1	13± 3	1.1×10^6	6,7
RD	12± 7	1.0×10^6	6,7
HEL	4± 2	3.4×10^5	7

[a] Average fluorescent cell count represents the average a single scan through the center of six different coverslip cultures.
[b] Virus assayed by the ICC procedure.
[c] Same number indicates no significant difference at 95 percent confidence interval.
ICC Assay Multiplication Factors:
$X10^2$ - The viruses were diluted 100 fold.
X16.8 - Factor for a single scan through the center of the coverslip culture.
X50 - 0.02 ml inocula were used (0.02 = 1/50).

Table 4 shows our cumulative results from comparing cell line sensitivities to the three reovirus serotypes.

Sensitivity of MDBK, LLC-MK_2 and BGM Cells to Protamine Precipitated Reovirus

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TABLE 4. SENSITIVITY OF VARIOUS CELL LINES TO REOVIRUS SEWAGE ISOLATES

Cell Line	Duncan's Multiple Range Test Ranking and Average Cell Count					
	Reovirus Type 1		Reovirus Type 2		Reovirus Type 3	
	Statistical[a] Ranking	Average Cell Count[b]	Statistical[a] Ranking	Average Cell Count[b]	Statistical[a] Ranking	Average Cell Count[b]
MDBK	1	337	1	249	1	118
LLC-MK_2	2	181	2	199	1	123
Int. 407	3	132	3	182	2	106
BGM	4	111	4	160	5,6	21
MDCK	5	66	7,8	51	2,3	98
MA-104E	5	60	5	124	4	72
Vero	6	44	6	100	6	16
BS-C-1	6	43	6	94	6,7	13
PK	6	39	8	41	5	27
BHK-21	7	15	7	56	3	93
RD	7	5	9	1	6,7	12
HEL	7	4	9	4	7	4

[a]Identical number indicated no significant difference between respective cell lines with 95 percent confidence intervals.

[b]Average fluorescent cell count represents the average of a single scan through the center of six different coverslip cultures.

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The purpose of this study was to determine which cell line was most susceptible to reoviruses that had not had previous cell contact. The sources of these viruses are given in the Methods section. From the previous data, MDBK and LLC-MK_2 cells were the most sensitive to the three reovirus serotypes. BGM cells were tested because they have been reported to be especially useful in water virology. The intestinal 407 cells, although sensitive, would not withstand the toxic conditions of the protamine sulfate inoculum so were not used in the experiments.

The ICC assay was used without dilution of the inoculum and one-half the coverslip was counted. Results of this study are shown in Table 5.

MDBK cells were the most sensitive when compared with LLC-MK_2 and BGN cells. The Duncan's Multiple Range comparisons detected a significant difference between MDBK cells and LLC-MK_2 and BGM cells.

TABLE 5. SENSITIVITY OF MDBK, LLC-MK_2 AND BGM CELL LINES TO REOVIRUS PRECIPITATED FROM SEWAGE BY PROTAMINE SULFATE

Isolate Sample	Cell Line	Average Cell[a] Count and Standard Deviation	Infectivity[b] titer/liter of Raw Sewage	Duncan's Mult. Range Compar.[c]
A	MDBK	60±15	4.2×10^4	1
	LLC-MK_2	12± 2	8.4×10^3	2
	BGM	13± 6	9.1×10^3	2
B	MDBK	43± 7	3.0×10^4	1
	LLC-MK_2	11± 3	7.7×10^3	2
	BGM	16± 4	1.1×10^4	2
C	MDBK	5± 2	3.5×10^3	1
	LLC-MK_2	2± 1	1.4×10^3	1,2
	BGM	1± 1	7.0×10^2	2

[a]Average cell count represents the average number of infected cells counted on half of six different coverslip cultures.
[b]Virus assayed by an ICC procedure.
[c]Same number indicates no significant difference at 95 percent confidence interval.

ICC Assay Multiplication Factors:
X7 - The viruses in a liter of raw sewage were concentrated to 7 ml. Multiplying by 7 equals the number of viruses if the sewage had been concentrated to 1 ml.
X2 - One-half of each coverslip culture was counted.

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X50 - 0.02 ml inocula were used. 0.02 = 1/50 of a milliliter. ICC/liter = 2(ICC count of 1/2 culture) x 7 x 50.

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Comparison of the Plaque and Immunofluorescent Cell Count (ICC) Assays

MDBK cells were used to compare the efficiencies of plaque and ICC assays in detecting reoviruses precipitated from sewage by the modified protamine sulfate procedure (Table 6).

The ICC assay was more sensitive with less variation in counts. The ICC titers were 2 to 12 fold higher, respectively, than the plaque assay titer in the first and second experiments. Also, the plaque counts in both experiments included a very high percentage (41 percent) of non-reovirus plaques (refer to next experiment).

TABLE 6. COMPARISON OF IMMUNOFLUORESCENT CELL COUNT (ICC) ASSAY USING PROTAMINE SULFATE PRECIPITATED REOVIRUS WITH TOTAL PLAQUE COUNTS ON MDBK CELLS.

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Experiment Number	Assay Method	Dilution Assayed	Average[a] Cell Count and Standard Deviation	Infectivity[b] titer/liter of Raw Sewage
1	ICC	10^{0}	43±7	3.0×10^{4}
	Plaque	10^{-1}	44±15	1.5×10^{4}
2	ICC	10^{0}	37±8	2.6×10^{4}
	Plaque	10^{0}	59±26	2.1×10^{3}

[a]ICC - fluorescent cells/0.5 coverslip - average cell count represents six different coverslip cultures.
Plaque assay - plaque forming units (PFU)/25 cm^2 flask.
Average cell count represents three data points.

[b]Multiplication factors for:
1) The ICC assay.
   X7 - The viruses in a liter of raw sewage were concentrated to 7 ml/1. Multiplying by 7 equals the number of viruses if the sewage had been concentrated to 1 ml.
   X2 - One-half of each coverslip culture was counted.
   X50 - 0.02 ml inocula were used. 0.02 = 1/50 of a milliliter ICC/liter = 2(ICC count of 1/2 culture) x 7 x 50
2) The Plaque assay.
   X - The dilution factor - (10^{-1})

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X7 - See X7 in (1) above.
X5 - 0.2 ml inocula were used. 0.2 - 1/5 of a milliliter.
PFU/liter - PFU of culture x dilution factor x 7 x 5.

Identification of the Plaque Isolates that were Reoviruses

A number of plaques observed on MDBK, LLC-MK_2, and BGM cells were picked and assayed on MDBK cells by the ICC assay method. The MDBK cell sheets contained a uniform distribution of plaques and, therefore, could be quantitatively compared to the ICC assay (Table 6). Plaques on BGM and LLC-MK_2 cell sheets could not be compared due to extensive cell sheet destruction. The percentage of non-reovirus plaques was lowest in MDBK cells (41 percent) and highest in BGM cells (69 percent) (Table 7). Three of the six LLC-MK_2 cell plaques contained reoviruses.

TABLE 7. IDENTIFICATION OF PLAQUE ISOLATES FROM PROTAMINE PRECIPITATED SEWAGE

Cell Line	Total No. of Plaques Isolated	Reovirus Plaques	Non-Reovirus Plaques	% of Non-Reovirus Plaques
BGM	13	4	9	69
LLC-MK_2	6	3	3	50
MDBK	34	20	14	41

Discussion

The purpose of this study was to find continuous cell lines that are sensitive to sewage borne reoviruses of the three serotypes. Consequently, many of the cells used in the comparative assays were of primate origin.

Monkey kidney cell lines (especially the BGM cell line) have gained wide use in water virology. Dahling et al. (3) report BGM cells are more sensitive than primary Rhesus or African green monkey kidney cells for recovery of viruses from water. However, Schmidt et al. (17,18) contend BGM cells are unsatisfactory and excel in sensitivity only with poliovirus and group B coxsackievirus. MDBK, MDCK, intestinal 407, PK, and BHK-21 cells have had minimal, if any, use in water virus isolation work. Wecker and Meulen (27) have shown good sensitivity of RD and HEL cell lines to enteroviruses.

The superiority of the MDBK cell line in detecting the serotyped reoviruses that had been previously grown in monkey kidney cells, and also the protamine-precipitated virus that had

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no cell passage history, was unexpected. The MDBK cells were as sensitive as any of the primate cells even when the reoviruses to which they were exposed had been isolated and cultivated in monkey kidney cells. Also, since the reoviruses obtained from California were isolated in large metropolitan areas, it is unlikely they are bovine reovirus. Therefore, the intestinal 407 cells, a human embryonic intestinal cell, should have demonstrated good susceptibility. Evidently the human or non-human origin of the viruses and the cell passage history did not influence the sensitivity of the cells. The sensitivity of the LLC-MK_2 cells would be consistent with the results of Schmidt et al. (17), since the LLC-MK_2 cells (which were obtained in their tenth passage) would be more similar to primary monkey kidney cells than the four other monkey kidney cells used since they had been passaged extensively.

The BGM cell line, which the Duncan's Multiple Range Test ranked fourth in sensitivity to reovirus types 1 and 2, and fifth to reovirus type 3, was infected with protamine-precipitated reovirus to further assess its susceptibility to sewage reoviruses. In the ICC assay, the cells remained unaffected by the protamine sulfate in the inocula, but gave low counts in comparison with MDBK cells. The performance of BGM cells in the plaque assay was similar to that of LCC-MK_2 cells, i.e., there was extensive degradation of cell sheets, which is unsatisfatory for a reovirus detection procedure. Using BGM cells, Dahling et al. (3) demonstrated a sensitivity to reovirus type 1 unsurpassed by primary monkey kidney cells. Schmidt et al. (18) observed a greater number of "false" plaques in BGM cells than in the other cells tested. Some waste water samples apparently contain small particulate toxic materials, which (when localized by the solid overlay) resemble virus plaques, but from which virus is not recoverable. Pseudoplaques and other enteric viruses could explain the 69 percent nonreovirus plaques obtained from BGM cells (Table 7). The above results show that BGM cells are inferior to MDBK cells for the isolation of reoviruses from sewage.

The sensitivity for reoviruses of the other eight cell lines used in the initial study (MDCK, MA-104E, Vero, BS-C-1, PK, BHK-21, RD, and HEL) were too low to warrant further research. Immunoflorescence in infected HEL and BHK-21 cells was less intense in the ICC assay due to the fibroblastic morphology. The other six cell lines, being epithelial-like, produced easily read cell sheets.

The plaque and the ICC methods were compared to determine which was best for assaying reoviruses in sewage. The plaque assay has been widely used and recommended for the enumeration, identification, and isolation of viruses. Schmidt et al. (18) and Nupen (15), however, cited the roller tube culture method under

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fluid media as being more sensitive than the plaque assay under semi-solid media. Katzenelson (8), in comparing the plaque assay and an ICC assay using poliovirus, believed the ICC assay had the rapidity necessary for viral identification in potable waters. In our comparison with the ICC assay, using reovirus as the indicator virus, the plaque assay was found to have limitations. For example, the optimum incubation time for reovirus detection by the fluorescent antibody procedure is 20-22 hr, although detection is possible within 10 hr (21). This rapid identification-enumeration is crucial for a procedure used by water renovation plants. In comparison, the formation of plaques requires 4-7 days and the identification of plaques as "false" or viral requires considerably more time.

The short reovirus detection time of the ICC assay insures considerably less than usual contamination in the host cell cultures. During the initial stages of plaquing, fungizone and gentimicin had to be added to the overlay media to inhibit fungi and bacteria in contaminated cell sheets. The ICC assay had a much lower incidence of contamination and in many cases where contamination did occur, the fluorescent cells could still be enumerated and the viruses quantified.

The quantification of reoviruses not only needs to be rapid but also accurate and sensitive. The ICC assay identified more reoviruses from sewage, with less variation in counts, than did the plaque assay (Table 6). This became more significant when it was observed that 41 percent of the plaques isolated on MDBK cells represented non-reovirus plaques (Table 7).

In this study, the protamine sulfate concentration method effectively concentrated reoviruses from polluted waters. This procedure has many characteristics that are advantageous for the development of a routine test for virus polluted waters. It can be performed in three hours, requires no expensive equipment, and recovers naturally-occurring reoviruses in titers in excess of 10^5 infectious particles/liter of raw sewage. (See Section 5 of this report). Melnick et al. (12) refer to total enteric viral concentration in sewage of approximately 5×10^5/liter. Table 6 shows reovirus titers in excess of 10^4/liter in raw sewage.

In conclusion, reoviruses have the characteristics required for a water pollution test virus. In other studies we have detected the virus in titers exceeding 10^5 infectious particles/liter of raw sewage, and other researchers throughout the world find it in abundance. Reoviruses have not been shown to cause overt diseases in humans and, therefore, resemble Escherichia coli in not being likely targets of any eradication program. Satter and Westwood (16), sampling a water renovation plant, detected less reduction in the concentration of reovirus present in water after chlorination than in concentrations of the other enteric viruses. Reoviruses thus appear to be highly resistant to

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inactivation. The results of this present research demonstrated the sensitivity of MDBK cells for isolating endogenous reovirus and the rapidity of the immunofluorescent cell count assay method for the detection of reoviruses in less than 24 hours.

The superiority of the MDBK cell line for detecting naturally-occurring reoviruses is economically advantageous for water renovation analysis. Bovine kidney cells are considerably less expensive and more available than primate cells. We have successfully infected MDBK cells that have a passage number of over 150 without losing sensitivity. The MDBK cells, in comparison with other cell lines, are relatively easy to grow and maintain. Finally, when analyzing polluted waters, MDBK cells are less affected by the usual toxic constituents than are the primate cell lines.

We recommend the use of MDBK cells in conjunction with a primate kidney cell, such as LLC-MK_2, for the detection of reovirus and other enteric viruses in water.

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PROTAMINE PRECIPITATION OF TWO REOVIRUS PARTICLE TYPES

Sonication Studies of Reovirus in River Water

9-1/8"

Suspensions of reovirus in river water were sonicated to disrupt viral aggregates. It appeared that, rather than breaking up aggregates, the sonication converted the PIV to IV and then inactivated the IV (Figure 1). If viral aggregates had been dispersed, graphs of the infectivity assays of IV and PIV would both show initial increases in virus numbers. During the first half minute of sonication, the IV was inactivated and the PIV resisted inactivation. The sonication evidently loosened the outer coat of about 90 percent of the PIV particles and converted them to IV particles during the next half minute of sonication. This is shown by a loss of PIV and an increase in IV. After all of the convertable PIV was changed to IV, the IV was inactivated. About 10 percent of the PIV resisted conversion to IV. Controls remained unchanged in titer throughout the experiments. These results indicated that our stock virus was not appreciably aggregated and would therefore permit a more than usual precision in evaluating the protamine precipitation method.

Protamine Sulfate Concentration of Reovirus from River Water

England's protamine sulfate procedure (4) was used to precipitate IV and PIV added to river water. In these initial experiments, a heavy protamine floc was produced which rapidly plugged the AP20 filters. An additional problem was encountered when cultures were inoculated with the dissolved protamine precipitate. A cloudy film formed and interfered with fluorescent antibody

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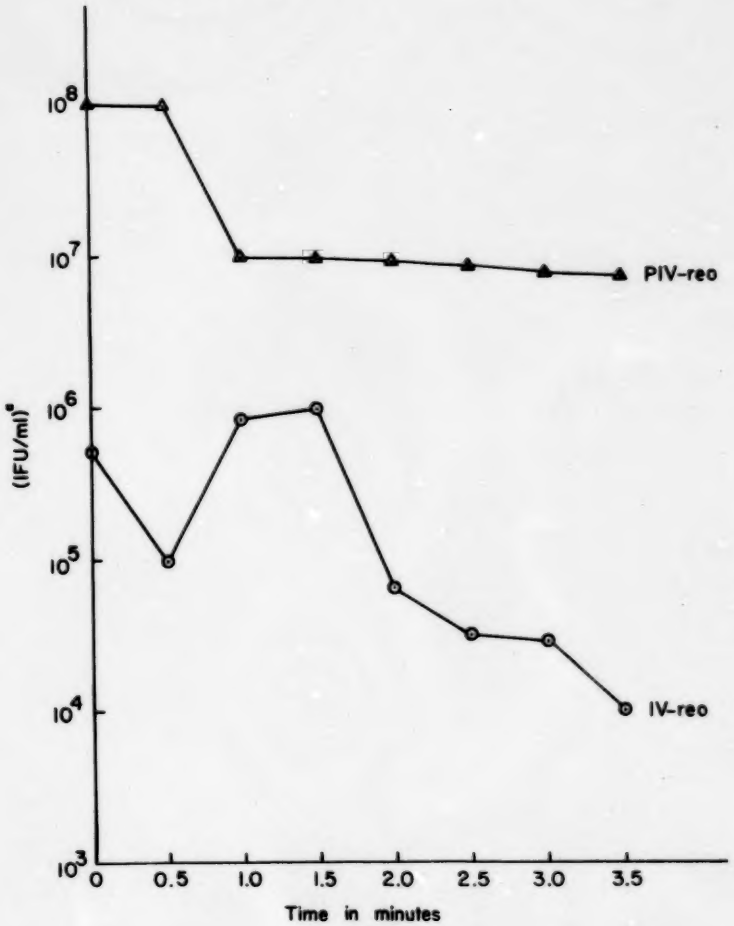

[a]ICC determined by fluorescent cell counts.

Figure 1. Effects of sonication on reovirus. IV -- sonicated virus that was assayed before enzyme treatment. PIV -- sonicated virus that was assayed after enzyme treatment. Results represent averages of two experiments. Virus controls (IV and PIV) without sonication were assayed at each time interval and their titers remained unchanged throughout the experiments.

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staining used in the viral assay. Consequently, the concentrations of the reagents used in the protamine precipitation procedure were varied. Fetal bovine serum at 0.25 percent and protamine sulfate at 0.005 percent for the first precipitation, and 0.0025 percent for the second precipitation, resulted in clear coverslips for assay, and precipitated between 64 and 86 percent of the IV. In these and other protamine experiments, virus concentration and efficiency of virus recovery were related (Table 8). The best viral recoveries were obtained when virus concentrations were low. Controls were run in which the virus was not precipitated by protamine, i.e., virus was added to filtered river water, mixed for the duration of the experiment, filtered, and then assayed before and after chymotrypsin treatment. Under the conditions of those experiments (Table 8), the PIV was apparently masked by the protein present in the precipitate. Less than 1 percent of the IV and PIV fractions were detected in the filtrate.

Percentages of recovery of IV (Table 8) were increased by 5 to 15 percent when two equal volumes of 1 M NaCl were used to elute the protamine precipitate from the AP20 disc instead of one elution with the same total volume. Each volume of 1 M NaCl eluent was left on the filters for 5 minutes before the eluent and dissolved protamine precipitate were collected by vacuum. The filter was then rinsed with 6 volumes of sterile water to achieve isotonic conditions in the filtrate.

Chymotrypsin Treatment of Reovirus before Concentration with Protamine

In attempts to determine why both IV and PIV virus fractions were not recovered in the protamine precipitation procedure (see Table 8), the reovirus stock was treated with 20 µg/ml chymotrypsin before adding it to the filtered river water. This converted the PIV to IV. In this experiment, 79 to 95 percent of the total virus added was recovered (Table 9). The control in these experiments was chymotrypsin-treated virus added to water, mixed for the duration of the experiment without protamine, filtered through an AP20 disc, then assayed before and after a second chymotrypsin treatment of 20 µg/ml. As expected, the PIV that had been converted to IV with enzyme prior to being added to the river water was recovered.

Increasing the Chymotrypsin Concentration to Detect PIV

Table 10 shows the results of varying the amounts of chymotrypsin used to enhance the infectivity of the viral preparations. The best recovery was obtained using 200 µg of chymotrypsin per milliliter, which is ten times the concentration previously used. The efficiency of recovery again seems to be related to the viral

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TABLE 8. INFLUENCE OF VIRUS CONCENTRATION ON REOVIRUS RECOVERY FROM WATER BY PROTAMINE PRECIPITATION

ICC Added to[a] River Water	ICC Recovered in Concentrate	Recovery (Percent)	Variance ± Standard Devia.	Reovirus Particle Form
5.1×10^4_7 (w/o ct)	4.4×10^4_4 (w/o ct)	86	16.6 ± 4.1	IV
1.0×10^7 (ct)	4.4×10^4 (ct)	<1		PIV
5.2×10^5_8 (w/o ct)	3.9×10^5_5 (w/o ct)	75	38.0 ± 6.2	IV
1.1×10^8 (ct)	3.8×10^5 (ct)	<1		PIV
5.2×10^6_9 (w/o ct)	3.5×10^6_6 (w/o ct)	67	24.0 ± 4.9	IV
1.0×10^9 (ct)	3.2×10^6 (ct)	<1		PIV
$5.3 \times 10^7_{10}$ (w/o ct)	3.4×10^7_7 (w/o ct)	64	56.9 ± 7.5	IV
1.2×10^{10} (ct)	3.3×10^7 (ct)	<1		PIV

ICC = Immunofluorescent cell count.
(w/o ct) = Virus precipitated with protamine and assayed without chymotrypsin treatment.
(ct) = Virus precipitate assayed after treatment with 20 μg/ml of chymotrypsin for 1 hour at 37°C. Four different virus concentrations were added to filtered river water and precipitated with protamine sulfate with subsequent assay by fluorescent cell counts.

[a]After reovirus was added to filtered river water, the water was mixed for the duration of the experiment with protamine sulfate, filtered, and then assayed before and after chymotrypsin treatment. Experimental controls were treated in a similar manner except that the protamine was not added.

TABLE 9. CHYMOTRYPSIN TREATMENT OF REOVIRUS (CONVERSION OF PIV TO IV) PRIOR TO PRECIPITATION WITH PROTAMINE SULFATE

ICC Added to[a] River Water	ICC Recovered in Concentrate	Recovery (Percent)	Variance ± Standard Deviation	Reovirus Particle Form
7.7×10^5 (ct-1)	7.3×10^5 (ct-1)	95	6.2 ± 2.5	IV
7.6×10^5 (ct-2)	7.3×10^5 (ct-2)	93	5.0 ± 2.2	PIV
1.1×10^7 (ct-1)	8.7×10^6 (ct-1)	79	10.7 ± 3.3	IV
1.0×10^7 (ct-2)	8.6×10^6 (ct-2)	84	26.0 ± 5.1	PIV

Virus was initially treated with 20 µg/ml of chymotrypsin for 1 hour at 37°C, added to filtered river water, and precipitated with protamine sulfate.

ICC = Immunofluorescent cell count.
ct-1 = Virus assayed after protamine precipitation before a second chymotrypsin treatment.
ct-2 = Virus assayed after protamine precipitation and a second chymotrypsin treatment.

[a]After chymotrypsin treated reovirus was added to filtered river water, the water was mixed for the duration of the experiment with protamine sulfate, filtered, and then assayed before and after a second chymotrypsin treatment. Experimental controls were treated in a similar manner except that the protamine was not added.

TABLE 10. DETECTION OF PIV IN THE PROTAMINE PRECIPITATE BY INCREASING THE CHYMOTRYPSIN CONCENTRATION

Chymotrypsin Concentration (g/ml)	ICC Units Added to[a] River Water	ICC Units Recovered in Concentrate	Recovery (Percent)	Variance ± Standard Devia.	Reovirus Particle Form
20	9.3×10^6 (w/o ct)	6.3×10^6 (w/o ct)	68	28.2 ± 5.3	IV
	8.5×10^8 (ct)	6.1×10^6 (ct)	<1		PIV
40	9.2×10^6 (w/o ct)	6.4×10^6 (w/o ct)	69	30.9 ± 5.6	IV
	8.5×10^8 (ct)	6.2×10^6 (ct)	<1		PIV
60	9.4×10^6 (w/o ct)	4.4×10^6 (w/o ct)	47	122.0 ±11.0	IV
	8.4×10^8 (ct)	4.3×10^6 (ct)	<1		PIV
80	9.3×10^6 (w/o ct)	2.9×10^6 (w/o ct)	31	142.9 ±12.0	IV
	8.4×10^8 (ct)	2.7×10^6 (ct)	<1		PIV
100	9.2×10^6 (w/o ct)	3.8×10^6 (w/o ct)	41	88.2 ± 9.4	IV
	8.7×10^8 (ct)	3.5×10^6 (ct)	<1		PIV
100[b]	4.6×10^5 (w/o ct)	3.7×10^4 (w/o ct)	80	13.55± 3.7	IV
	4.3×10^5 (ct)	3.8×10^4 (ct)	9	26.0 ± 5.1	PIV
150[c]	4.6×10^5 (w/o ct)	3.9×10^4 (w/o ct)	85	18.7 ± 4.3	IV
	4.2×10^5 (ct)	4.3×10^4 (ct)	10	23.2 ± 4.8	PIV
200	9.3×10^6 (w/o ct)	7.4×10^6 (w/o ct)	80	10.5 ± 3.2	IV
	8.5×10^8 (ct)	8.4×10^8 (ct)	99	1.19± 1.1	PIV
200[d]	4.5×10^5 (w/o ct)	4.0×10^5 (w/o ct)	89	17.68± 4.2	IV
	4.3×10^5 (ct)	4.3×10^5 (ct)	100	0.18± 0.4	PIV

ICC = Immunofluorescent cell count units.
(w/o ct) = Virus precipitated with protamine assayed without chymotrypsin treatment.
(ct) = Virus precipitated with protamine and assayed after chymotrypsin treatment.

[a]After reovirus was added to filtered river water, the water was mixed for the duration of the experiment with protamine sulfate, filtered, and then assayed before and after chymotrypsin treatment. Experimental controls were treated in a similar manner except that protamine was not added.

[b,c,d]Observations taken from a second set of experiments using a different viral stock containing fewer IV and PIV.
The IV and PIV comparisons in this table are made horizontally, not vertically.

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concentration. The control for these experiments was the same as in the previous two experiments except that 200 µg/ml of chymotrypsin was used prior to assay of the PIV. These results suggested that the protamine or the serum interfered with the enzymatic enhancement of the viral infectivity. Chymotrypsin was used at 200 µg/ml in all subsequent assays.

Stability of Reovirus Fractions in the Protamine Precipitate

The stability of reovirus in protamine precipitates was investigated to determine the feasibility of precipitating viruses from water in the field and transporting the precipitate to the laboratory. The viral concentrate, with 5 percent FBS, was stored in glass tubes after elution from the prefilter. The stability of both IV and PIV in the protamine precipitates is shown in Table 11. The results show that the samples can be stored at -20°C or 4°C for at least 18 days with only a small loss of infectivity.

Concentration of Reovirus from Raw Sewage

The modified protamine concentration procedure and the fluorescence assay method were used to concentrate and assay 10 raw sewage samples from Utah sewage treatment facilities (Table 12). Five samples from Salt Lake City, and five samples from Dugway, Utah were collected. Three of the five samples collected at Salt Lake City contained detectable IV. In two of these samples, virus was also detected after enzyme treatment, at the same or lower concentration than without enzyme treatment. The IV concentrations ranged from 2-5 x 10^3/l. Four out of five samples collected at Dugway contained reoviruses. Three samples contained detectable IV ranging in concentration from 0.32 - 1.2 x 10^5/l. In one sample, PIV alone was detected at a concentration of 2.1 x 10^5/l. These results indicate that different concentrations of chymotrypsin might be needed to accurately assay the PIV in different sewage samples.

Discussion

In this report, we describe a modified protamine sulfate procedure for precipitating reoviruses from polluted water, and a fluorescent antibody method for assaying the precipitated virus. The procedures concentrate reoviruses from 4 liters to 8 ml, recover between 80 and 100 percent of IV and PIV fractions, and produce quantitative data within 24 hours.

Application of these techniques to raw sewage successfully recovers both the IV and PIV. Inconsistent results are sometimes obtained when the sewage precipitates are treated with chymotrypsin. In experiments using chymotrypsin to increase the infectivity of laboratory-produced virus, the infectivity of IV was not lost. With some of the sewage isolates, however, the IV and PIV, if

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TABLE 11. STABILITY OF REOVIRUS IN THE DISSOLVED PROTAMINE PRECIPITATE STORED AT 4°C AND -20°C

Storage Conditions[a]	Storage Time (Days)	ICC Units Remaining in Concentrate	Percent of Initial Viral Fraction Remaining Infectious	Reovirus Particle Form
	0	2.0×10^6 (w/o ct)	100	IV
		2.0×10^8 (ct)	100	PIV
	4	2.0×10^6 (w/o ct)	100	IV
		2.0×10^8 (ct)	100	PIV
4°C	14	2.4×10^6 (w/o ct)	120*	IV
		1.6×10^8 (ct)	80	PIV
	18	2.6×10^6 (w/o ct)	130*	IV
		1.6×10^8 (ct)	80	PIV
	0	2.0×10^6 (w/o ct)	100	IV
		2.0×10^8 (ct)	100	PIV
	4	2.0×10^6 (w/o ct)	100	IV
		2.0×10^8 (ct)	100	PIV
20°C	14	2.1×10^6 (w/o ct)	105*	IV
		1.9×10^8 (ct)	95	PIV
	18	2.1×10^6 (w/o ct)	105*	IV
		1.8×10^8 (ct)	90	PIV

ICC = Immunofluorescent cell count units.
(w/o ct) = Virus assayed without chymotrypsin treatment.
(ct) = Virus assayed with chymotrypsin treatment at 200 µg/ml for 1 hour at 37°C.

[a]Protamine precipitated samples were eluted from the prefilters, and stabilized with 5 percent fetal bovine serum low in reovirus antibody.

*Conversion of PIV to IV during storage.

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present, were lost upon treatment with 200 µg/ml of chymotrypsin. This could be caused by any of several factors including heterogeneity in the components of raw sewage, the virus existing in a semi-weakened state, (having altered outer and/or inner capsids) or the presence of CT^- reovirus. Chymotrypsin treatment of the CT^- reoviruses inactivates rather than enhances the infectivity of the virus (23).

A range of chymotrypsin from 50 µg to 300 µg/ml should be considered when enhancing the infectivity of reoviruses precipitated from sewage. In addition, all samples should be assayed before and after chymotrypsin treatment.

Our recovery of more than 10^5 infectious reoviruses per liter of untreated sewage (Table 12) suggests that the modified protamine precipitation and fluorescent antibody staining procedures can be useful in determining the potential roles of the IV and PIV in water quality testing. England (4) pointed out that volumes which can be concentrated with this type of procedure fall short of what is needed to detect minimal pollution in recreational and culinary waters, but would be effective when run in parallel with or in conjunction with other large-volume concentration procedures. However, the protamine procedure could possibly be used with large volumes of water by collecting the precipitates on pleated filters, or by tangential flow filtration.

Reports indicate incoming virus loads in raw sewage ranging from several thousand to several hundred thousand per liter (7). Without examination of other virus populations at the locations checked in our study, reoviruses cannot be assumed to be the most abundant animal virus present. Nevertheless, their occurrence in these systems is evident. The results from Dugway, Utah also indicate that the viral concentrations in undiluted raw sewage might be higher than previously expected.

During storage of the protamine concentrates, there was an increase of 5 percent in the IV and a 10 percent loss of the PIV after 18 days at -20°C. Both fractions showed more pronounced changes when stored at 4°C; i.e., the IV increased 30 percent and the PIV decreased 20 percent. These changes might be due to a conversion of the PIV to IV or a combination of events including antibody reactions and disruption of aggregates. Regardless, these results show that it would be feasible to precipitate reoviruses from water and only bring the protamine-virus precipitate to the laboratory.

It would be premature at this time to speculate about the overall presence or absence of PIV in polluted waters. A more extensive investigation of waters and wastewaters is needed, with emphasis on the stability of IV and PIV in sewage. Wastewaters should be examined for the presence of both the adsorbed and free IV and PIV fractions to further evaluate reoviruses as an indicator of viral pollution in the environment.

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TABLE 12. PROTAMINE SULFATE PRECIPITATION OF REOVIRUS NATURALLY OCCURRING IN RAW SEWAGE

Location	Sewage[a] Sample	(ICC/1)[c] Recovered from Raw Sewage	Reovirus Particle Form
Dugway, Utah	1	<400 (w/o ct)	IV
		2.1×10^5 (ct)	PIV
	2	<400 (w/o ct)	IV
		<400 (ct)	PIV
	3	1.2×10^5 (w/o ct)	IV
		<400 (ct)	PIV
	4	1.5×10^5 (w/o ct)	IV
		<400 (ct)	PIV
	5[b]	3.2×10^4 (w/o ct)	IV
		3.0×10^4 (ct)	PIV
Salt Lake City, Utah	1	<400 (w/o ct)	IV
		<400 (ct)	PIV
	2	2.0×10^3 (w/o ct)	IV
		1.6×10^3 (ct)	PIV
	3	4.0×10^3 (w/o ct)	IV
		4.0×10^3 (ct)	PIV
	4	<400 (w/o ct)	IV
		<400 (ct)	PIV
	5	5.0×10^3 (w/o ct)	IV
		<400 (ct)	PIV

ICC = Immunofluorescent cell count units
(w/o ct) = Virus precipitated with protamine and assayed before chymotrypsin treatment
(ct) = Virus precipitated with protamine and assayed after chymotrypsin treatment using 200 µg/ml for 1 hour at 37 °C

[a] Two 1,000 ml samples were used for each determination. The average is recorded in the table. Final processed sample volumes were 8.0 ml.

[b] This sewage sample was stored for 3 weeks at -20° before precipitation and assay.

[c] 400 ICC/1 represents the detection limit of the immunofluorescent assay as used in this study, employing an assay volume of 20 µl.

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A PROTAMINE SULFATE CONCENTRATION METHOD FOR REOVIRUSES AND ROTAVIRUSES ADAPTABLE TO LARGE WATER VOLUMES

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Preliminary Studies

In preliminary studies using the procedure developed by England (4), the protamine floc facilitator was found to cause a viscous precipitate to form which limited the amount of sample that could be trapped on a prefilter. Fetal bovine serum used in the protamine procedure was found to contain high levels of rotavirus neutralizing antibody which renders the procedure ineffective for rotavirus. The use of egg albumin circumvents the above problems.

Trypsin Treatment of Reovirus and Rotavirus

Early work on a protamine precipitation procedure for reovirus and rotavirus established that the infectivity of both viruses could be significantly increased with trypsin treatment. Table 13 shows results obtained when reovirus and bovine rotavirus stocks were trypsin treated. The infectivity titers were increased 13.6 times with reovirus and approximately 50.8 times with rotavirus. Often, maximum increases in virus infectivity in tissue culture fluids could be achieved with trypsin concentrations of 10 µg/ml of sample. As Table 13 shows, up to 200 µg trypsin/ml of sample can be employed to achieve maximum virus infectivity enhancement.

Trypsin at this concentration was effective in dispersing the protamine precipitate. However, trypsin concentrations below 100 ug/ml did not significantly disperse the protamine precipitate, or produce maximum infectivity increases of virus concentrated by this method.

TABLE 13. TRYPSIN ENHANCEMENT OF REOVIRUS AND BOVINE ROTAVIRUS INFECTIVITY[a]

Trypsin Concentration µg/ml	Reovirus/ml	Rotavirus/ml	Virus Particle Assayed
0	4.4×10^6	6.3×10^5	IV
10	6.0×10^7	3.2×10^7	IV/PIV
20	5.8×10^7	3.0×10^7	IV/PIV
40	6.0×10^7	3.0×10^7	IV/PIV
100	7.4×10^7	3.2×10^7	IV/PIV
200	7.4×10^7	3.0×10^7	IV/PIV
500	4.7×10^7	2.5×10^7	IV/PIV
1000	3.9×10^7	2.4×10^7	IV/PIV

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[a]Stocks were trypsin treated in MEM without FBS and inoculated onto confluent coverslip cultures of MDBK cells after trypsin neutralization with soybean trypsin inhibitor.

Trypsin treatment of virus in the presence of the protamine precipitation reagents was used for determining titers of virus controls in all experiments.

Modified Protamine Sulfate Precipitation Procedure

The protamine sulfate precipitation procedure outlined in Table 14 uses egg albumin as a precipitate facilitator. The optimum concentration of egg albumin is somewhat dependent upon the organic content of the water source. Upon incubating the protamine-albumin-sample- mixture for 30 min at 37°, with occasional mixing, a precipitate is formed which can be collected by centrifugation at 2000 X g for 15 minutes. The supernatant is discarded and the precipitate is dispersed with 1 M NaCl. Distilled water is added to make the solution isotonic. An aliquot of isotonic solution is then treated with 200 ug of trypsin/ml of sample followed by enzyme neutralization and assay with a rapid immunofluorescent antibody technique.

TABLE 14. SCHEME FOR PRECIPITATION OF REOVIRUS AND ROTAVIRUS WITH PROTAMINE SULFATE AND EGG ALBUMIN

I Precipitation	add egg albumin to polluted water to 0.12 to 0.14% final concentration
	adjust the pH to 8.5 - 8.8
	add protamine sulfate to 0.01% final concentration
	incubate 30 min at 37°C with occasional mixing
	centrifuge at 2,000 x G for 15 min
Discard Supernatant	

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II Elution of virus	resuspend precipitate in a small volume of 1M NaCl
	mix well to disperse precipitate
	add 6 volumes of distilled water and mix thoroughly (isotonic conditions)
	treat with 200 µg trypsin/ml sample for 30 min at 37°C
	add trypsin inhibitor to sample for assay of combined IV and PIV particles
	infectivity assay

Protamine Precipitation of Reovirus and Rotavirus Added to Water and Sewage

The efficiency of the modified protamine sulfate precipitation method was studied by inoculating water samples with various concentrations of reovirus and bovine rotavirus. Stocks of both viruses containing from 1×10^4 to 1×10^7 ICC/ml were independently added to samples of 0.2 to 1.0 l of chlorine-free water and precipitated as outlined in Table 14. Variations in recovery of virus were greater between different experiments than in the same experiments in which different concentrations of virus inoculums and sample volumes were used. Therefore, virus inocula of approximately 5×10^6 ICC/ml and sample volumes of 0.2 l were used for all subsequent experiments. Each sample was assayed on MDBK cells and represents combined IV/PIV virus populations. Table 15 shows that highest virus recoveries were obtained from water samples spiked with reovirus and rotavirus. Lowest virus recoveries were obtained when these viruses were precipitated from secondary sewage which had not been filtered to remove suspended solids. Recoveries of approximately 90 percent were obtained when reovirus or rotavirus was precipitated from raw sewage or secondary sewage without suspended solids. Statistical analysis using a Duncan's Test at the 95 percent confidence level, showed no significant difference between recoveries of inoculated virus from the various water and wastewater samples.

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TABLE 15. PROTAMINE SULFATE PRECIPITATION OF REOVIRUS AND ROTAVIRUS ADDED TO WATER[a] AND SEWAGE

Sample	Virus Added	Number of Experiments[d]	Average Recovery %	Recovery Range %
Water	Reovirus	9	94	88 - 100
Sewage[b] without Suspended Solids	Reovirus	6	90	84 - 100
Sewage[c] with Suspended Solids	Reovirus	6	86	79 - 95
Raw Sewage	Reovirus	6	90	83 - 100
Water	Rotavirus	9	95	89 - 100
Sewage without Suspended Solids	Rotavirus	6	91	79 - 100
Sewage with Suspended Solids	Rotavirus	6	88	80 - 95
Raw Sewage	Rotavirus	6	89	82 - 98

[a] No chlorine residual.
[b] Secondary Effluent minus suspended solids removed by filtration
[c] Secondary Effluent containing 10-20 mg/l suspended solids.
[d] Each experiment consisted of 3 replications using 200 ml of sample containing approximately 5×10^6 ICC of virus/ml. Final processed sample volumes were 3.5 ml. Assay volumes were 20 µl.

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Protamine Sulfate Precipitation of Naturally Occurring Reovirus

The protamine precipitation procedure was used to concentrate naturally occurring reoviruses from raw sewage for a period of three months (Table 16). The viral concentrates obtained were assayed three ways: (a) undiluted before trypsin treatment, (b) undiluted with trypsin treatment, and (c) diluted 1:10 followed by trypsin treatment. Initially a 1:10 dilution without trypsin was also assayed, but the results are not listed because infected cells were seldom detected. The undiluted samples assayed before and after trypsin treatment showed approximately the same recoveries. However, the trypsin treated samples had an overall higher recovery range. The samples diluted 1:10 and assayed after trypsin treatment showed a higher recovery average of 1.0×10^4 ICC/l. Four recoveries out of 22 total samples collected assayed greater than 1×10^5 ICC/l. Also, no samples collected during this three month time interval were negative.

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Discussion

Early investigations by England (4) and our current study have demonstrated the suitability of protamine sulfate techniques for the concentration of reoviruses from sewage. England suggested that reoviruses might be the most frequently present and abundant animal virus in sewage (4). In section 5B of this report, over 1×10^5 reovirus ICC units per liter in raw sewage were reported.

There is mounting epidemiological evidence that rotavirus can be transmitted through the consumption of sewage-polluted waters (10,14). However, because of the lack of suitable methodology, rotaviruses could not be demonstrated in the incriminated water samples. Therefore, we considered it important to modify the protamine procedure so it would recover reoviruses and rotaviruses. The present study has shown that the modified technique developed is highly efficient in concentrating both reoviruses and a bovine rotavirus.

In this modified protamine precipitation procedure, viral concentrates are trypsin treated to assay both IV/PIV virus fractions. Although the PIV fractions of both reoviruses and bovine rotavirus in infectious culture fluids is usually 10 to 100 fold higher in concentration than the IV, the presence of PIV particles in polluted waters has not been determined.

The mechanism of trypsin enhancement of both reovirus and rotavirus infectivity and of the additional infectivity enhancement resulting from exposure of the viruses to protamine, egg albumin and salt is unknown. However, work in our laboratory (unpublished data) found that trypsin acts on the virus and not the infected cell.

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TABLE 16. PROTAMINE SULFATE PRECIPITATION OF REOVIRUSES OCCURRING NATURALLY IN RAW SEWAGE[a]

Sample Date	Sample Volume (ℓ)	ICC/ℓ[b] recovered from raw sewage: Undiluted without trypsin	Undiluted[c] with trypsin	1:10 dilution[c] with trypsin
6/13/79	0.5	not done	1.2×10^4	3.0×10^4
6/18/79	1.5	not done	1.3×10^4	1.3×10^5
6/22/79	1.0	not done	3.6×10^3	1.4×10^4
6/27/79	1.0	not done	1.9×10^4	2.1×10^4
7/02/79	1.0	not done	3.2×10^4	8.1×10^4
7/06/79	1.0	not done	++	9.5×10^4
7/09/79	1.0	not done	1.1×10^5	2.9×10^5
7/11/79	0.5	3.9×10^4	3.5×10^4	3.9×10^4
7/13/79	1.0	4.2×10^4	2.9×10^4	4.2×10^4
7/16/79	1.0	8.6×10^4	++	1.7×10^5
7/20/79	1.0	6.9×10^4	1.2×10^5	1.9×10^5
7/23/79	1.0	3.7×10^4	3.8×10^4	4.8×10^4
7/25/79	1.0	8.8×10^4	3.8×10^4	9.5×10^4
7/27/79	1.0	9.0×10^3	7.6×10^3	8.9×10^3
8/01/79	1.0	4.9×10^4	5.0×10^4	5.3×10^4
8/03/79	1.0	2.0×10^4	2.8×10^4	not done
8/06/79	1.0	1.1×10^3	1.8×10^3	9.6×10^3
8/09/79	1.0	5.3×10^4	4.7×10^4	not done
8/17/79	1.0	8.8×10^3	8.8×10^3	9.6×10^3
8/21/79	1.0	++	++	5.0×10^4
8/30/79	1.0	++	3.4×10^2	No detectable virus
9/05/79	1.0	4.2×10^3	4.3×10^3	8.7×10^3

[a]All samples were collected from Hyrum, Utah sewage treatment plant at the point of entry between 10:30 and 12:30 p.m. Final processed sample volumes were 3.5 ml for 0.5ℓ volumes and 7 ml for volumes of 1.0ℓ and 1.5ℓ.

[b](ICC/ℓ) Immunofluorescent cell count units/liter. Assay volumes of 20 µl were used.

[c]Prior to assay samples were treated with 200 µg trypsin per ml of sample for 30 min. at 37°C followed by addition of 300 µg of soy bean trypsin inhibitor per ml of sample before inoculation onto confluent coverslip monolayers of MDBK cells. Counts are adjusted to undiluted concentration.

++Poor cell sheets made assay impossible.

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In developing a protamine precipitation procedure to concentrate and assay reovirus and rotavirus infectivity using a rapid immunofluorescent antibody technique, several different grades of egg albumin were tried as protamine floc facilitators. Preliminary experiments using raw egg whites (prepared in the laboratory), and commercially available grade II and III egg albumin showed that carefully collected egg whites (without yolk material) or grade III egg albumin produced stocks which resulted in consistent virus recoveries. The inconsistency of commercially available grade II egg albumin made testing of each lot of grade II albumin necessary before it was used in experiments. For the sake of consistency, grade III albumin was used in all experiments.

Past protamine procedures (4) and the procedure described previously on page 8 of this report have suffered from volume limitations caused by collection of the viral precipitate on filter surfaces. An important advantage of our modified procedure is that it can be scaled-up to sample large quantities of water, thus increasing the probability of recovering viruses when they are present in low concentrations. Concentration and assay of small volumes (up to 1 liter) of raw sewage were handled effectively without removing debris from the sewage. However, it became apparent on the larger volumes that a preliminary centrifugation step at 2000 x g for 15 min to remove most of the suspended solids, was desirable. If larger sewage samples are not centrifuged the amount of solids concentrated by the procedure interferes with the virus infectivity assay. Since protamine sulfate may react with naturally occurring proteinaceous substances present in water, the optimal concentration of egg albumin required to achieve maximum virus recoveries was found to vary from 0.12 to 0.14 percent final concentration. We found 0.12% egg albumin to be the optimal final concentration for the Hyrum treatment facility.

Protamine precipitation of various water and sewage samples spiked with reovirus and/or rotavirus resulted in very good recoveries. Average recoveries ranged from a high of 94 to 95 percent in water to from 86 to 88 percent in secondary effluent with suspended solids present. The decrease in recoveries when viruses were precipitated from sewage samples may have been due to virus adsorption to the particulates. Results were the lowest when finely divided particles were in suspension as in the sewage samples that were not filtered or centrifuged before the viruses were added.

Protamine precipitation successfully recovered over 1 x 10^5 ICC of reovirus per liter of raw sewage. Reports have been made of incoming virus loads in raw sewage ranging from several thousand to several hundred thousand per liter (7). The results of our three month study at Hyrum, Utah showed average reovirus recoveries to be 5.0 x 10^4 ICC/l. Without examination of the other virus populations at this location, reoviruses cannot be

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assumed to be the most abundant animal virus present, but their abundance in these systems is evident.

In summary, the modified protamine sulfate precipitation procedure developed in this study effectively precipitates reoviruses and rotaviruses from diverse waters. The viral precipitates can be collected by continuous flow centrifugation procedures, so large volumes of water can be processed and quantitative data can be obtained in less than 24 hours. Also, using this modified precipitation procedure, reoviruses were found in high concentrations in raw sewage. These observations coupled with the ubiquitous nature of reoviruses suggest that reoviruses show considerable promise as indicators of waterborne enteric virus pathogens.

COMPARISON OF THE CONCENTRATION OF REOVIRUSES AND ENTEROVIRUSES IN SEWAGE

Raw sewage from Hyrum, Utah was concentrated by organic flocculation and assayed for reoviruses and total enteroviruses from 6/24/80 to 5/28/81 (Fig. 2). Reoviruses without trypsin treatment were isolated on MDBK cells and detected by immunofluorescence while total enterovirus counts were obtained by plaquing with BGM cells. Endogenous reovirus concentrations averaged 1.6×10^4/liter and ranged from 1.7×10^3 to 7.6×10^4/liter. Endogenous enterovirus concentrations averaged 2.6×10^3/liter and ranged from 5.4×10^2 to 9.8×10^3, although on one occasion (12/30/80), no enteroviruses could be detected due to the highly toxic nature of the sample. Both reovirus and total enterovirus concentrations exhibited high variability from sample to sample and no significant trends in their concentrations throughout the study period were apparent. Also, no correlation between endogenous reovirus and enterovirus levels in raw sewage was evident, i.e., there was no simultaneous seasonal increase or decrease in concentration of the two viral groups. These results demonstrate that average reovirus recoveries in Hyrum, Utah sewage were 6.2 fold greater than total enterovirus levels and that reovirus could be detected in every sample during the study.

Raw sewage from Ogden (Table 17) and Salt Lake City, Utah (Table 18) was monitored for the presence of reoviruses and total enteroviruses from 9/4/80 to 2/24/81. Endogenous reoviruses were detected in 12/12 Ogden samples and averaged 5.3×10^3 per liter with a range of 1.7×10^3 to 1.6×10^4/liter. Enteroviruses were detected in all but three Ogden samples. The high concentration was 4.4×10^4 PFU/ml. The average enterovirus concentration could not be calculated because of the negative samples.

Sewage samples from Salt Lake City, Utah were obtained from a treatment plant serving industrial as well as residential areas.

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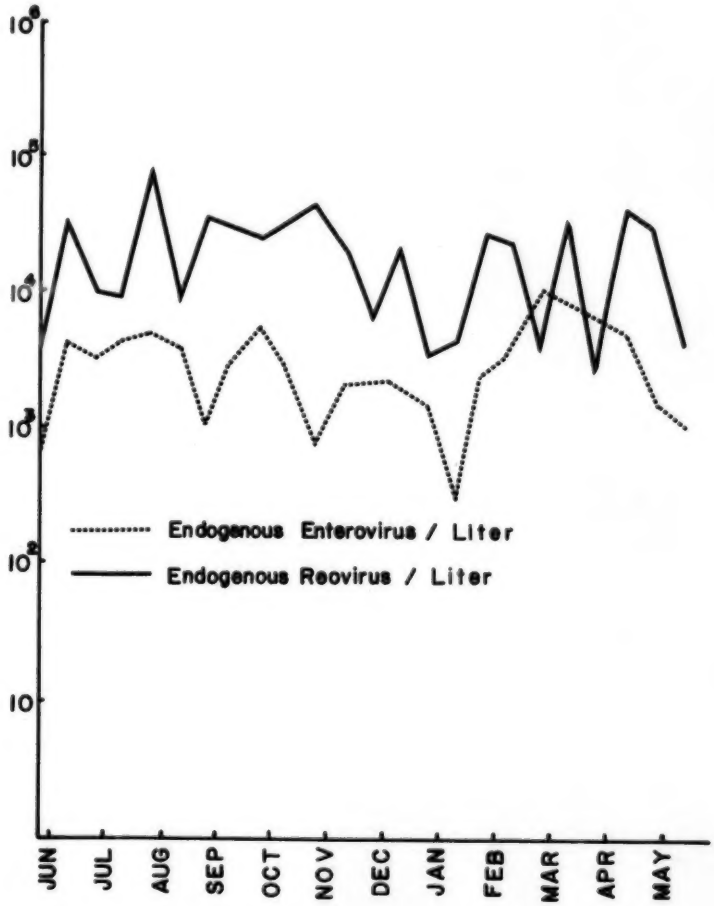

Figure 2. Relative concentrations of reoviruses and enteroviruses in Hyrum, Utah raw sewage.

Consequently, these samples were highly toxic to cells, even when diluted in tissue culture fluids. Thus, 10 of 12 samples were negative for reoviruses and 4 of 12 were negative for enteroviruses. When relatively low toxicity was apparent, both reoviruses and enteroviruses were detected, which highlights the need for development of an assay which can be used to detect viruses in sewage originating from industrial areas.

TABLE 17. CONCENTRATIONS OF REOVIRUSES AND ENTEROVIRUSES IN OGDEN, UTAH SEWAGE

Sample Date	Endogenous Reoviruses ICC/liter	Reovirus "Spike" Recovery (%)	Endogenous Enteroviruses PFU/liter	Poliovirus "Spike" Recovery (%)
9/4/80	7.4×10^3	102	7.0×10^2	106
9/24/80	4.9×10^3	88	3.2×10^3	100
10/1/80	1.6×10^4	105	1.0×10^3	108
10/16/80	3.4×10^3	100	9.5×10^2	68
10/30/80	3.0×10^3	100	1.3×10^4	140
11/13/80	2.7×10^3	88	3.0×10^3	107
12/10/80	1.1×10^4	108	$<2.2 \times 10^2$	91
12/31/80	2.8×10^3	82	broken tube	
1/15/81	1.7×10^3	80	4.5×10^3	150
1/27/81	3.4×10^3	85	4.4×10^2	80
2/10/81	2.2×10^3	72	$<2.2 \times 10^2$	18
2/24/81	5.0×10^3	90	$<2.2 \times 10^2$	contaminated

TABLE 18. CONCENTRATIONS OF REOVIRUSES AND ENTEROVIRUSES IN SALT LAKE CITY, UTAH SEWAGE

Sample Date	Endogenous Reoviruses ICC/liter	Reovirus "Spike" Recovery (%)	Endogenous Enteroviruses PFU/liter	Poliovirus "Spike" Recovery (%)
9/4/80	$<5.6 \times 10^2$	72	$<2.2 \times 10^3$	67
9/24/80	$<5.6 \times 10^2$	68	2.7×10^3	115
10/1/80	$<5.6 \times 10^3$	52	1.0×10^3	70
10/16/80	3.0×10^2	93	$<2.2 \times 10^3$	55
10/30/80	$<5.6 \times 10^2$	67	$<2.2 \times 10^3$	160
11/13/80	$<5.6 \times 10^2$	64	1.2×10^3	90
12/10/80	$<5.6 \times 10^2$	57	$<2.2 \times 10^3$	62
12/31/80	$<5.6 \times 10^2$	71	3.4×10^3	55
1/15/81	$<5.6 \times 10^3$	53	2.8×10^2	114
1/27/81	1.1×10^2	73	$<2.2 \times 10^4$	97
2/10/81	$<5.6 \times 10^2$	77	7.3×10^4	67
2/24/81	$<5.6 \times 10^2$	81	4.3×10^4	88

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Microtiter Optimization Results

Optimization studies were carried out to increase the sensitivity of the microtiter ICC assay. The results of these experiments are presented in Tables 19-21. Sensitivity increased with increasing speed of centrifugation and decreasing inoculum volume (Table 19). Maximum sensitivity was achieved with 250 ul of inoculum centrifuged at 3000 rpm, the minimum volume of inoculum which sufficiently covered cell sheets and the maximum speed of centrifugation tolerated by microtiter plates. An approximately 13-fold increase in sensitivity was observed when the inoculum was centrifuged at 3,000 when compared to the uncentrifuged control. Using the optimal conditions of inoculum volume and speed of centrifugation, 250 ul and 3,000 rpm, respectively, the optimal time of centrifugation (virus adsorption period) was determined to be 1 hour (Table 20). Centrifugation for 1.5 hours did not result in a significant change in sensitivity.

Next, the sensitivity of the standard coverslip procedure used in our laboratory was compared to the sensitivity of the microtiter plate assay (Table 21) utilizing the optimal conditions determined in the previous studies. These results show that the microtiter plate assay is approximately 2 fold more sensitive than the coverslip assay when optimal conditions of inoculum volume (250 ul), speed (3,000 rpm) and time (1 hour) of centrifugation are employed.

TABLE 19. OPTIMIZATION OF SPEED OF CENTRIFUGATION AND INOCULUM VOLUME FOR ASSAY OF REOVIRUS BY THE IMMUNOFLUORESCENT CELL COUNT METHOD

Speed of Centrifugation (rpm)[a]	Inoculum Volume (ul) 250	500	1000
None[b]	1.7×10^{6}[c]	9.2×10^{5}	5.0×10^{5}
1,000	4.2×10^{7}	3.0×10^{6}	1.6×10^{6}
2,000	1.1×10^{7}	6.1×10^{6}	3.3×10^{6}
3,000	2.2×10^{7}	1.4×10^{7}	6.0×10^{6}

[a] 24-well microtiter plates were centrifuged in a Sorvall GLC-1 tabletop centrifuge for 1/2 hour at room temperature.
[b] A 24-well microtiter plate was inoculated and placed in a 37°C incubator for a 1/2 hour adsorption period.
[c] Reovirus titer per ml in MDBK cells assayed by the ICC cell count method.

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TABLE 20. OPTIMIZATION OF TIME OF CENTRIFUGATION FOR ASSAY OF REOVIRUS BY THE MICROTITER ICC

Time of Centrifugation (hr)[a]	Reovirus Titer (ICC/ml)[b]
0.5	5.9×10^7
1.0	9.8×10^7
1.5	8.0×10^7

[a]24-well microtiter plates were inoculated with 250 µl of Freon-extracted reovirus in tissue culture fluids and centrifuged at 3,000 rpm in a Sorvall GLC-1 tabletop centrifuge at room temperature.
[b]Reovirus titer in MDBK cells assayed by the ICC

TABLE 21. COMPARISON OF THE SENSITIVITY OF 24-WELL MICROTITER LOW-SPEED CENTRIFUGAL INOCULATION TO THE STANDARD COVERSLIP PROCEDURE FOR ASSAY OF REOVIRUS BY THE ICC PROCEDURE

Inoculum Volume (ul)	Reovirus Titer (ICC/ml)[d]
250[a]	4.9×10^7
200[a]	5.2×10^7
100[a]	5.6×10^7
50[a]	6.4×10^{7b}
20[a]	1.8×10^{8b}
20[c]	2.4×10^7

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[a]24-well microtiter plates were inoculated with Freon-extracted reovirus in tissue culture fluids and centrifuged for 1 hour at 3,000 rpm in a Sorvall GLC-1 tabletop centrifuge at room temperature.
[b]Only a ring of infected cells around the periphery of the microtiter wells could be detected due to the low inoculum volume used, therefore, these results were considered to be unreliable.
[c]15 mm circular glass coverslip cell cultures were inoculated with 20 µl of Freon-extracted reovirus in tissue culture fluids, placed in a 37°C incubator for a 1 hour adsorption period, and assayed by the immunofluorescent cell count method.
[d]Reovirus titer in MDBK cells assayed by the ICC.

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Discussion

Recovery of endogenous reoviruses averaged 6.2 fold higher than endogenous enterovirus levels over a one-year sampling period in Hyrum, Utah sewage. In Odgen, Utah sewage, endogenous reovirus concentrations were approximately equal to total enterovirus concentrations. In a recent study, reoviruses comprised 39% of all viruses isolated from sewage in Australia and were present on several occasions when enteroviruses could not be detected (6). These studies indicate that reoviruses are consistently present and usually comprise the majority of endogenous virus recovered from sewage. Thus, reoviruses should be considered as "indicator viruses" or at least included in any standardized procedure used to estimate the viral pathogenic potential of waters.

Viruses were isolated only occasionally from Salt Lake City, Utah sewage due to the highly toxic nature of sewage originating from industrialized localities.

During the course of this study, the conditions of the microtiter plate ICC assay were optimized to enhance the sensitivity of the reovirus detection procedure. The optimal conditions for assay of reoviruses were determined to be 250 µl of inoculum, and centrifugation at 3,000 rpm (1,500 x g) for one hour. However, endogenous reovirus levels in sewage reported in this study were determined using 500 ul of inoculum with centifugation for 1/2 hour at 2,000 rpm. Thus, according to the results presented in Table 19, endogenus reovirus concentrations may have been 3.6 fold higher than the levels reported in this study.

By centrifuging inocula at 3,000 rpm, the sensitivity of the ICC was increased by approximately 13-fold when compared to an uncentrifuged control. The increase in sensitivity observed when low-speed centrifugal inoculation is employed could be due to a cell effect, such as an increase in available virus adsorption sites or, as suggested by Schoub et al. (19), the concentration of heavy viral aggregates or virus particles attached to organic matter onto the cell surface.

In order to assess the importance of enzyme enhanceable reoviruses (PIV) as an indicator of viral pollution in water, sewage samples from Hyrum, Ogden and Salt Lake City were assayed for endogenous reoviruses with and without trypsin treatment. A composite of these results is presented in Table 22. In most cases, no significant difference was observed between reovirus titers treated or not treated with trypsin, which suggests that the PIV particles in sewage were proteolytically enhanced by bacterial and/or mammaliam proteolytic enzymes present in the sewage. However, in several instances, trypsin treatment of sewage extracts resulted in either significant increases or decreases in endogenous reovirus titers. Cliver and Herrman (1) reported that several human enteroviruses were inactivated by

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bacterial and mammalian proteases likely to be found in sewage, so it is not surprising to find degradation as well as enhancement of reovirus infectivity by proteolysis.

The major difference between the results reported in Tables 16 and 22 was the loss of infectivity reported in the latter experiments when some of the preparations were treated with trypsin. In the earlier study, the virus was precipitated using protamine sulfate. In the subsequent study the beef extract procedure, which exposes the virus to a pH of 3.5, was used. Enzyme treated virus may not be as stable as untreated virus at this low pH.

TABLE 22. A COMPOSITE OF RESULTS OBTAINED WHEN SEWAGE SAMPLES WERE ASSAYED FOR ENDOGENOUS REOVIRUS (PIV AND IV)

Sample Location	Sample Date	Reovirus Titer (ICC/liter) w Trypsin (PIV)[a]	Reovirus Titer (ICC/liter) w/o Trypsin (IV)	PIV/IV[b]
Hyrum	1/14/81	3.9×10^3	1.1×10^4	0.4
Hyrum	4/28/81	5.9×10^3	1.7×10^3	3.5
Ogden	12/31/80	5.6×10^3	2.8×10^3	2.0
Ogden	1/15/81	5.6×10^2	1.7×10^3	0.3
Salt Lake City 1	1/27/81	5.6×10^2	1.1×10^3	0.5
Salt Lake City 2	2/10/81	2.8×10^4	5.6×10^3	5.0

[a] Sewage extract was incubated for 30 minutes at 37°C in the presence of 100 µg/ml trypsin and subsequently trypsin activity was stopped with soybean trypsin inhibitor before infection of cell cultures and assay for endogenous reovirus by the ICC assay as described in materials and methods.

[b] The ratio of reovirus titers obtained with (potentially infectious virus, PIV) and without (infectious virus, IV) trypsin treatment.

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